

Skeletochronological Study on Age Structure of a Chinese Endemic Frog (*Rana omeimontis*)

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Abstract We used skeletochronology to estimate age structure of the Omei Wood Frog, *Rana omeimontis*, from southwestern China. Average age differed significantly between males and females. Age at sexual maturity in both males and females was 1 year. Longevity was 6 and 7 years for males and females, respectively. Average body size differed significantly between the sexes, with females being larger than males. A non-significant correlation between age and body size was found within each sex in this population. The ANCOVA analysis revealed that females also had larger body size than males when the effect of age was controlled.

Keywords skeletochronological method, *Rana omeimontis*, age structure, sexual size dimorphism

1. Introduction

Skeletochronology has proven to be a reliable determinant of an individual's age in amphibians due to the presence of lines of arrested growth (LAGs) recorded in cross-sections of the long bones in phalanges since the 1970s (Castanet and Smirina, 1990). This method has been successfully demonstrated in assessing age in several temperate (Hemelaar, 1980; Lu *et al.*, 2006; Chen *et al.*, 2011; Liao, 2011), desert (Sullivan and Fernandez, 1999), subtropical (Lai *et al.*, 2005; Li *et al.*, 2010; Liao and Lu, 2010 a, b, c; Liao *et al.*, 2011; Liao and Lu, 2011; Yang *et al.*, 2011; Liu *et al.*, 2012; Lou *et al.*, 2012) and tropical anuran species (Guarino *et al.*, 1998; Khonsue *et al.*, 2001).

Age and body size are two demographic traits important for understanding the evolutionary life history and ecology of anurans (Morrison and Hero, 2003; Liao and Lu, 2011). Currently, many anurans are experiencing catastrophic population declines all over the world due

to global temperature warmer, ultraviolet-B (UV-B) radiation, habitat disappearance and human construction (Beebe and Griffiths, 2005). Therefore, demographic data are necessary for effective conservation planning for anuran populations (Morrison, 2001; Liao and Lu, 2012; Ma *et al.*, 2009).

The Omei Wood Frog, *Rana omeimontis*, is endemic to Chinese subtropical forests and is widely distributed within elevations of 250–2100 m. The breeding season of this frog lasts from late August to middle September with metamorphosis of the tadpoles occurring from following May to July (Fei *et al.*, 2005). Although some information on population distribution and reproduction of *R. omeimontis* has been published in recent years (Fei and Ye, 2001), surprisingly little is known about its age, body size and growth rate. The aim of this study was to estimate ages at sexual maturity, longevity and growth, understand the relationship between body size and age, and gain insight into the mechanisms determining sexual size dimorphism.

2. Materials and Methods

The field study was conducted in Caiba Town of Yibin City (28°47' N, 104°33' E, 281 m a.s.l.) in Sichuan,

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China. The study site was an artificial pond, being approximately 3 m long, 1.5 m wide and 2 m deep. The vegetation at the sampling sites is characterized by silver grass (*Miscanthus floridulus*), common bread wheat (*Triticum aestivum*), eucalyptus (*Eucalyptus robusta*) and Oriental Arborvitae (*Platycladus orientalis*). In May and November of 2011, a total of 127 frogs (63 males and 64 females) were collected by hand at night with an electric torch. For each captured individual, we measured body size (snout-vent length: SVL) to the nearest 0.01 mm with a caliper, and estimated sex by observing directly the secondary sexual characteristics (the nuptial pads in adult males and ova in adult females). The second digit of the longest toe of right hind limb was removed and stored in 10% neutral buffered formalin for later skeletochronological analysis. All the individuals were released at the location of initial collection after sampling.

To determine the age of all individuals, a standard skeletochronological study was conducted (Castanet and Smirna, 1990). We removed the skin and muscle tissues of each digit and decalcified the remaining bones in 5% nitric acid for 48 h. Then, we washed them in running tap water for 24 h. We stained the decalcified digits for 150 min in Harris's haematoxylin and rinsed with distilled water. Subsequently, we dehydrated these stained bones through successive ethanol stages for 1 h in each concentration. Tissues were embedded in small paraffin blocks. We cross-sectioned the diaphyseal region of each phalanx at a thickness of 13 μm and selected the smallest medullar cavity of the sections to examine LAGs with a LEITZ dialux 40 microscope, and photographed the best sections using a Motic BA300 digital camera mounted on a Moticam2006 light microscope at $\times 400$ magnification. As suggested by Lu *et al.* (2006) and Liao *et al.* (2010), we assumed that each LAG corresponds to an annual arrest of individual growth. Some frogs which were collected in November would be ready to hibernate, so we counted the outer margin of the bone as an additional 1.0 year. Endosteal resorption of long bones starts from the inner surface of the bone, enlarging the marrow cavities and eroding a portion of LAGs when frogs have completed their hibernation (Rozenblut and Ogielska, 2005). However, this most likely did not occur in this species because a Kastschenko Line (KL, the division line between the endosteal and periosteal zones; Rozenblut and Ogielska, 2005) existed in the sections of all the individuals.

We used Student's *t*-test to compare the differences in body size and age between males and females, and

the Kolmogorov-Smirnov test to identify age structure between the sexes. We assessed the interaction between age and sex using general linear models (GLMs) treating SVL as a dependent variable, and age and sex as fixed factors. To see significance of difference in body size between sexes when the effect of age was controlled, age was included as a covariate in a separate model. The correlation between body size and age for each sex was evaluated using linear regression. All probabilities were two-tailed, and the significance level was set at $\alpha = 0.05$. Means were given \pm SD. All statistical tests were performed using SPSS software version 13.0. The collection of all the frogs used in this study was permitted by Forestry Bureau of Yibin, Sichuan, China, and animal treatment followed all applicable institutions of the Animal Care Guidelines in China.

3. Results

The hematoxylin-stained cross-sections of phalanges showed a series of narrow concentric haematoxylinophilic rings or lines separated by wider layers of paler background with sparsely distributed osteocytes in all individuals (Figure 1). False lines were rarely observed, and they were not considered as true LAGs. Double lines were observed in four males and five females, but these lines must be counted as a single true LAG. Endosteal resorption affected only the first LAG, which was rarely observed in this study based on the occurrence of the KL.

Adult age of *R. omeimontis* ranged from 1 to 6 years in males, and from 1 to 7 years in females (Figure 2). However, there were no 4 year old males and females, and the number of males or females over 4 years was only one (Table 2). Age distribution differed significantly between males and females (Kolmogorov-Smirnov test: $D = 3.33$, $P < 0.001$) with males having a significantly older average age than females (Table 1; Student's *t*-test: $t = 5.63$, $P < 0.001$).

Average body size differed significantly between males and females, with females having larger size than males (Table 1; Student's *t*-tests: $t = 2.52$, $P = 0.013$). There was a significant difference in body size between the sexes for the group of two years (Table 2; Mann-Whitney *U*-test).

Table 1 Body size (SVL, mm) and age (yrs) of *R. omeimontis* between the sexes. Values are mean \pm SD.

	Male (n = 63)	Female (n = 64)	<i>t</i>	<i>P</i>
Body size (mm)	49.23 \pm 4.18	51.43 \pm 5.57	2.52	0.013
Age (yrs)	2.14 \pm 0.93	1.27 \pm 0.82	5.63	< 0.001

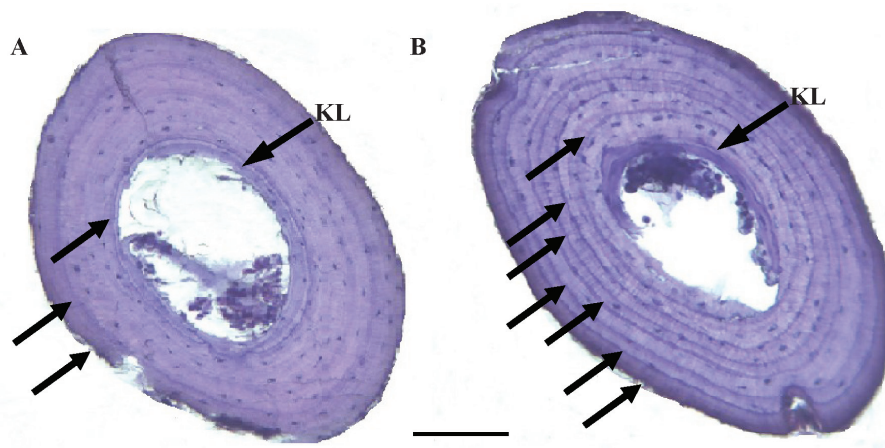


Figure 1 Two examples (A: a 3-yr old male; B: a 7-yr old female) of hematoxylin-stained cross-sections of the phalangeal bone of *R. omeimontis* from southwestern China. Arrows indicate the lines of arrested growth (LAGs). KL represents Kastschenko Line, the division line between endosteal and periosteal zones. Scale bar = 100 μ m.

The ANCOVAs analysis revealed that the difference in body size between the sexes remained significant ($F_{1, 124} = 11.68$, $P = 0.001$) when the effect of age was controlled ($F_{1, 124} = 6.17$, $P = 0.014$). A significant interaction between sex and age (ANCOVAs analysis: $F_{1, 119} = 13.67$, $P < 0.001$) revealed age-SVL relationship between the sexes differed in slope, suggesting that males and females have different growth pattern.

Linear regression analysis showed that non-significant relationship between age and body size was found within each sex (Figure 3; males: body size = $0.97 \text{ age} + 47.15$, $F_{1, 62} = 2.98$, $r = 0.22$, $P = 0.089$; females: body size = $1.54 \text{ age} + 49.48$, $F_{1, 63} = 3.39$, $r = 0.23$, $P = 0.07$).

Table 2 Difference in body size (SVL, mm) between the sexes within each age group of *R. omeimontis* from southwestern China. Values for male and female in descending order are means \pm SD, range and sample size.

Age class	Male	Female	Z	P
1	47.67 \pm 5.11 39.61–54.45 n = 14	49.88 \pm 4.46 33.59–59.37 n = 52	1.4	0.16
2	49.56 \pm 4.01 39.43–58.96 n = 31	59.04 \pm 4.07 55.48–68.58 n = 11	4.68	< 0.001
3	49.62 \pm 3.15 43.86–56.41 n = 16			
4	No data	No data		
5	46.39 n = 1			
6	57.34 n = 1			
7		48.41 n = 1		

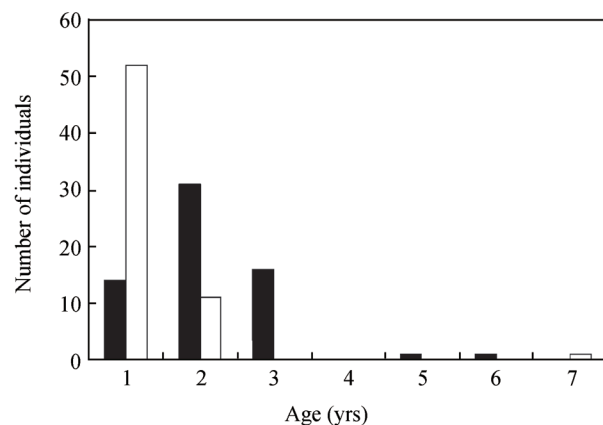


Figure 2 Adult age structure (male: close bars; female: open bars) in *R. omeimontis* from southwestern China.

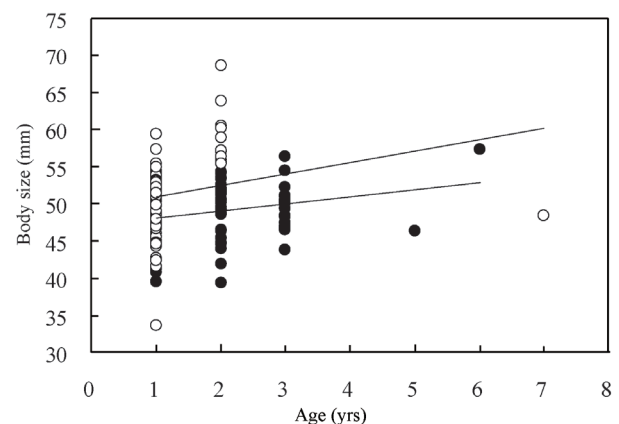


Figure 3 Relationship between body size and age (male: close circles; female: open circles) in *R. omeimontis* from southwestern China.

4. Discussion

Skeletochronology has been used for studies on anurans in a wide range of climate regimes (Monnet and Cherry, 2002). Our study showed that skeletochronology method can be successfully applied to *R. omeimontis* from southwestern China because of the clear arrest of growth in the hibernating period, as previously observed in other species in subtropical regions (Guarino *et al.*, 1998; Morrison *et al.*, 2004; Lai *et al.*, 2005; Liao and Lu, 2010c).

Age at maturity was estimated at 1 year in males and females. Sexual maturity at the same age for both sexes is found in other anurans, such as in *Pseudacris crucifer* (Lykens and Forester, 1987), *Hyla arborea* (Friedl and Klump, 1997), *R. epeirotica* (Tsiora and Kyriakopoulou-Sklavounou, 2002), *R. chensinensis* (Lu *et al.*, 2006), *R. ridibunda* (Kyriakopoulou-Sklavounou *et al.*, 2008), *R. nigromaculata* (Liao *et al.*, 2010), *R. limnocharis* (Liao *et al.*, 2011), *H. annectans chuanxiensis* (Liao and Lu, 2010 a), and *Bufo andrewsi* (Liao and Lu, 2012). However, for many amphibian species it is reported that females reach sexual maturity one year later than males, such as *R. temporaria* (Miaud *et al.*, 1999), *R. sylvatica* (Bastien and Leclair, 1992), *B. hemiophrys* (Eaton *et al.*, 2005), *Amolops mantzorum* (Liao and Lu, 2010 c), and *Hylarana guentheri* (Li *et al.*, 2010). In the present study, the lifespan of males and females are 6 and 7 years, respectively, suggesting that males tend to have a shorter longevity than females, which is consistent with most anurans (Khonsue *et al.*, 2001; Ento and Matsui, 2002; Matthews and Miaud, 2007; Li *et al.*, 2010). Differential mortality between sexes has been reported in frogs, where males exhibit more conspicuous behavior than females and can be easily detected by predators (Shirose *et al.*, 1993; Kyriakopoulou-Sklavounou *et al.*, 2008). This may be the reason why females live longer than males of *R. omeimontis*.

The age of *R. omeimontis* is not significantly correlated with body size within each sex. However, a positive significant correlation was found between age and body size for both sexes (Ryser, 1996; Lu *et al.*, 2006; Liao and Lu, 2010 c; Liao *et al.*, 2010; Liao and Lu, 2011) or one sex (Gibbons and McCarthy, 1984; Leclair and Castanet, 1987; Cherry and Francillon, 1992) in most anurans.

Like most amphibians, *R. omeimontis* is sexually dimorphic (Shine, 1979; Monnet and Cherry, 2002). Sexual size dimorphism may be the consequence of the differences in age (growth duration) (Monnet and Cherry, 2002; Liao and Chen, 2012) and post-maturation growth

rate (Howard, 1981) for both sexes. For this population, ANCOVAs revealed that age is a factor affecting sexual size dimorphism. However, males had a larger average age than females, which may have resulted from sampling since males are more abundant than females for the group of two and three years. Because males and females did not have a similar growth pattern, difference in growth rate between sexes would contribute marginally to the observed sexual size dimorphism. The resource allocation theory also may explain this observation in *R. omeimontis*. Male and female individuals differ in reproductive investment, so that similar environmental factors may impose different fitness consequence for male versus female life history (Faribairn, 1997; Schauble, 2004). Females need more stored energy, as they need to allocate much more energy to gonad and egg development than males do (Trivers, 1972). Males take more energy in reproduction rather than growth to improve the success of reproduction (Halliday and Tejedo, 1995). Moreover, *R. omeimontis* males have shorter longevity than females, so they rarely live long enough to attain a large body size (Shine, 1979).

Skeletochronology provides basic demographic data for natural populations of *R. omeimontis* that allows for future comparative and conservation studies. More detailed investigations on the variation in life-history traits in *R. omeimontis* are required in the future.

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